The lunatic fringe gene is a target of the molecular clock linked to somite segmentation in avian embryos

The *lunatic Fringe* gene is a target of the molecular clock linked to somite segmentation in avian embryos

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The most obvious segments of the vertebrate embryo are the trunk mesodermal somites which give rise to the segmented vertebral column and the skeletal muscles of the body. Mechanistic insights into vertebrate somitogenesis have recently been gained from observations of rhythmic expression of the avian *hairy*-related gene (*c-hairy1*) in chick presomitic mesoderm (PSM), suggesting the existence of a molecular clock linked to somite segmentation ([1]; reviewed in [2]). Here, we show that *lunatic Fringe* (*lFng*), a vertebrate homolog of the *Drosophila Fringe* gene, is also expressed rhythmically in PSM. The PSM expression of *lFng* was observed as coordinated pulses of mRNA resembling the expression of *c-hairy1*. We show that *c-hairy1* and *lFng* expression in the PSM are coincident, indicating that both genes are responding to the same segmentation clock. The genes were found to differ in their regulation, however; in contrast to *c-hairy1*, *lFng* mRNA oscillations required continued protein synthesis, suggesting that *lFng* could be acting downstream of *c-hairy1* in the clock mechanism. In *Drosophila*, Fringe has been shown to play a role in modulating Notch–Delta signalling [3,4], a pathway which in vertebrates has been implicated in defining somite boundaries [5–9]. These observations place the segmentation clock upstream of the Notch–Delta pathway during vertebrate somitogenesis.

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Results and discussion

Genetic studies in the mouse have implicated the Notch–Delta signalling pathway as playing a role in segmentation [10]. Mice harbouring mutations in the *Notch1*, *RBP-J kappa*, or *Delta1* genes retain a basic metamerism of paraxial mesoderm derivatives [5–7]. The somites in these mutants are, however, uncoordinated and their anterior–posterior compartments are incorrectly specified. Thus, the Notch–Delta pathway is thought to act later than the segmentation clock to both coordinate somite formation and specify boundaries during the segmentation process. Therefore, genes in this pathway are potential downstream targets of the segmentation clock; however, the chick genes *c-Notch1* and *c-Delta1* do not display rhythmic expression in the PSM [11]. A link between the two systems has yet to be established. To explore such a link, we analysed the expression of *lFng* during somitogenesis in the chick embryo. The *lFng* gene is expressed in the PSM [12–15] and has recently been shown to play a role in mouse somitogenesis [8,9]. We show that *lFng* transcripts are expressed in a rhythmic fashion in the PSM and thus appear to be directly regulated by the segmentation clock.

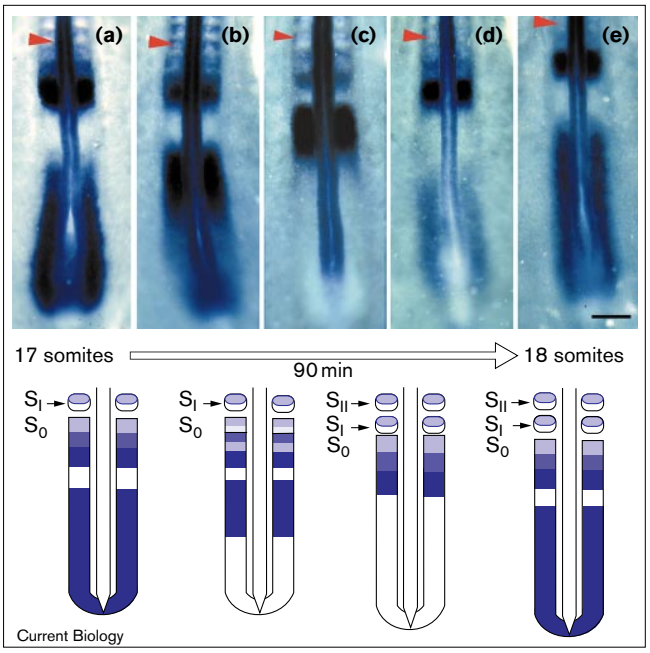
Expression of *lFng* is cyclical in the PSM

To analyse the expression of *lFng* in the PSM, a series of embryos ($n = 49$) were hybridised with an *lFng* probe. Expression of *lFng* was found to vary considerably even among embryos of the same somite number (Figure 1). Some embryos showed expression in two domains: a broad caudal domain extending from the tail bud region up to the rostral third of the PSM, and a narrower band located more rostrally in the PSM. In other embryos, the broad expression domain appeared to narrow, by becoming progressively downregulated caudally, while moving anteriorly. Concomitantly, the rostral-most domain became narrower and progressively faded in intensity. Weak expression was also observed within the anterior part of newly formed somites. Therefore, in the chick PSM, *lFng* displays a dynamic expression pattern.

In order to demonstrate the cyclical nature of this expression profile, the caudal portion of chick embryos was divided into two halves by sectioning along the midline; one half was fixed immediately, while the other half was cultured *in vitro* (Figure 2). When the experimental half was cultured for 30 minutes ($n = 15$), a very different profile of *lFng* expression was observed in each of the two halves (Figure 2a). This analysis enabled us to order the expression sequence of *lFng* in the PSM (Figure 1).

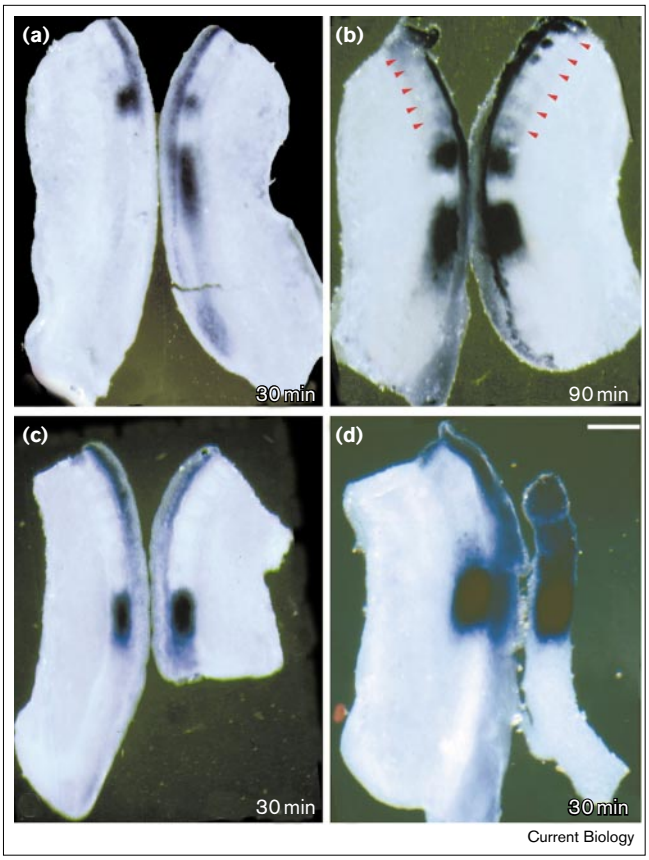
When the experimental half was incubated for 90 minutes ($n = 5$), a new somite was generated in the explant, and the two halves showed identical *lFng* expression patterns (Figure 2b). These experiments demonstrate that, like *c-hairy1*, *lFng* expression appears as a cyclical wavefront that sweeps along the PSM once during the formation of each somite.

Figure 1



Expression of *lFng* appears as a wavefront sweeping across the presomitic mesoderm. Upper panels show the dorsal view of the caudal region of **(a–d)** 17 somite stage and **(e)** 18 somite stage chick embryos hybridised with the *lFng* probe. In each panel, the last formed somite (S_I) is indicated by an arrowhead. Expression in the PSM appears as a broad caudal domain, which progressively moves anteriorly while narrowing as the caudal cleft of somite S_0 forms. The rostral domain corresponds to the residual expression domain of the previous wavefront (see panel e) and progressively fades in intensity as the caudal domain moves anteriorly. The rostral domain subsequently becomes anteriorly restricted in S_I. Rostral is to the top. Bar = 200 μm. The lower panel illustrates schematically the correlation between *lFng* expression in the PSM with the progression of somite formation. S_{II} indicates the last-but-one somite.

Figure 2



The *lFng* gene is expressed in a rhythmic fashion, correlating with somite formation, and is independent of adjacent structures. Caudal-half embryo explants were sagittally divided so that one half (left side) was either **(a,b)** fixed immediately or **(c,d)** cultured for the same time period as the right half. In all panels, both halves were hybridised with the *lFng* probe. (a) The right half was cultured for 30 min, and showed a different pattern of *lFng* expression from the left half. (b) When the right half was cultured for 90 min, a new somite formed, and the two halves showed identical *lFng* expression patterns. Red arrowheads indicate segmented somites. (c) The caudal part of the right explant was surgically removed and the remainder cultured in parallel with its contralateral half for 30 min. (d) Isolated PSM explant cultured for the same time period as the intact contralateral half (30 min). The expression pattern of *lFng* in the PSM is similar in operated and control halves in panels c and d. Rostral is to the top. The scale bar represents 260 μm in (a–c) and 130 μm in (d).

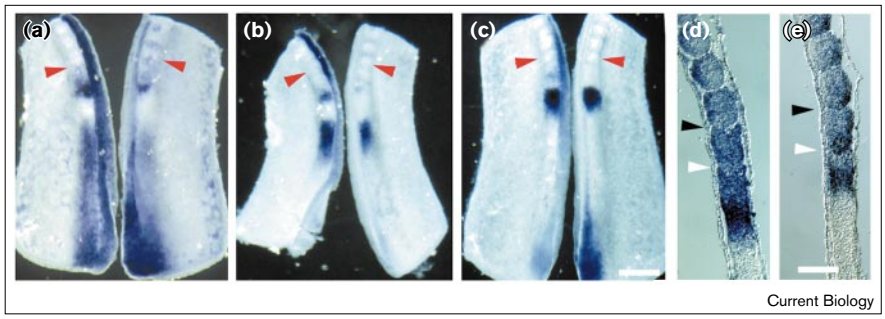
Cyclical expression of *lFng* is an autonomous property of the PSM

In order to analyse further the regulation of this expression pattern, we tested whether progression of the wavefront results from the propagation of a signal in the PSM. To that end, the expression of *lFng* was assayed in caudal-half embryo explants in which, on one side, the posterior part including the tailbud was surgically ablated. Both sides were then cultured for the same time. In all cases (n = 7), the same expression pattern of *lFng* was observed in the operated and control sides, indicating that the movement of the *lFng* wavefront does not rely on signal propagation (Figure 2c). This suggests that cyclical expression of *lFng* is an intrinsic property of the PSM. To verify this, we compared *lFng* expression in caudal-half embryo explants (n = 10) in which, on one side, the PSM was microdissected and isolated from the surrounding tissues. After culture under these conditions, the expression pattern in the isolated PSM was found to be the same as that of the intact side (Figure 2d). Therefore, like *c-hairy1*, rhythmic expression of *lFng* is an autonomous property of the PSM.

Tandem oscillation of *lFng* and *c-hairy1* in the PSM

A precise comparison of the *lFng* expression domain with that of *c-hairy1* in the PSM was performed in the caudal halves of chick embryos. Except in the rostral-most PSM, the expression domain of *lFng* on one side of the embryo was similar to that of *c-hairy1* on the other side (n = 20, Figure 3a–c). These findings indicate that the oscillations of both *c-hairy1* and *lFng* are coordinated in both time and

Figure 3



The expression domains of *lFng* and *c-hairy1* are similar in the PSM except in the rostral-most region. **(a–c)** Caudal portions of embryos were sagittally divided along the midline; each left half was hybridised with a *lFng* probe whereas each right half was hybridised with a *c-hairy1* probe. The two genes share the same expression domain in the PSM. Panels a, b and c show three different stages in the progression of the *c-hairy1* and *lFng* wavefronts in the PSM. Red arrowheads indicate somite S_I. **(d,e)** Alternate parasagittal sections hybridised with (d) *lFng* and (e) *c-hairy1*. The expression domains have clearly diverged along the forming somitic cleft. Black arrowheads indicate the rostral border of somite S_0. White arrowheads indicate the forming caudal boundary of somite S_0. Rostral is to the top. The scale bar in (c) represents 230 μm and applies to (a–c); the scale bar in (e) represents 115 μm and applies to (d,e).

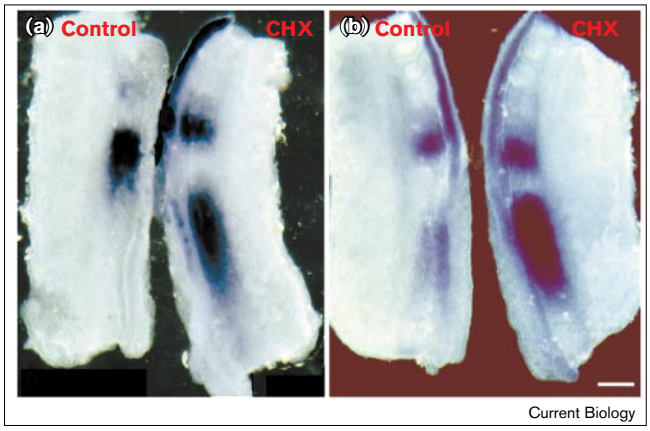
space and, thus, are controlled by the same segmentation clock. In the rostral-most PSM, in contrast, the expression domains of these two genes progressively diverge at the forming border of somite S_0 (Figure 3d,e).

Cycloheximide blocks *lFng* oscillations

The *c-hairy1* gene encodes a transcription factor of the basic helix-loop-helix (bHLH) family whereas *lFng* encodes a secreted molecule. As both genes are expressed with similar dynamics in PSM, one hypothesis might be that *lFng* acts downstream of *c-hairy1*. Such a model implies that translation of the *c-hairy1* product will be required in order to drive *lFng* expression. To address this idea, we examined whether blocking protein synthesis with cycloheximide affects *lFng* expression. Progression of the *lFng* wavefront was found to be retarded in cycloheximide-treated explants compared with the controls (10 out of 15 cases, Figure 4a). To confirm that the dynamic expression of *lFng* in PSM was indeed blocked, one explant half was fixed immediately while the other half was cultured for 30 minutes in the presence of cycloheximide. The expression pattern of *lFng* was similar on both sides, indicating a rapid and effective block of the *lFng* wavefront (9 out of 10 cases, Figure 4b). In similar experiments, *c-hairy1* expression was not affected ($n = 7$; data not shown, and [1]). These experiments show that the regulation of *c-hairy1* and *lFng* differ: both genes appear to be downstream of the segmentation clock, but only *lFng* requires protein synthesis to drive its dynamic expression. It is therefore possible that *lFng* acts downstream of *c-hairy1* in the PSM, although we cannot rule out the possibility that these genes are regulated by parallel pathways.

Our results demonstrate that *lFng* is a target of the recently identified segmentation clock, leading to periodic expression of *lFng* in the region where the prospective somite boundaries will form. In the fly, Fringe acts to differentially modulate Notch reception of Delta and Serrate ligands [3,4]. During vertebrate somitogenesis, Notch–Delta signalling is required for specification of the anterior and posterior somitic compartments, which occurs in the rostral-most PSM [10]. The *lFng* gene has also been shown to be essential for this process in the mouse [8,9]. The discrete expression domains of Notch and its ligands in this region where *lFng* oscillates suggests that, in the chick rostral PSM, *lFng* also modulates Notch signalling to establish the somite boundary. Thus, clock control on this local modulation of the Notch–Delta signalling pathway would confer the periodic arrangement of the boundaries that underlie the segmental body plan.

Figure 4



Dynamic expression of *lFng* in the PSM depends on *de novo* protein synthesis. **(a)** Half-embryo explants cultured for 30 min with (right side) or without (left side) cycloheximide (CHX). Expression of *lFng* differs between the control and CHX-treated sides. **(b)** The control explant (left) was fixed immediately while the right side was cultured for 30 min in the presence of cycloheximide. Expression of *lFng* was similar in each half. Note that cycloheximide treatment results in the stabilisation of mRNA transcripts within the expression domain [19]. Rostral is to the top. Bar = 140 μm.

Materials and methods

Eggs, embryos and somite nomenclature

Fertilised chick eggs (*Gallus gallus*, JA57, Institut de Sélection Animale, Lyon, France), obtained from commercial sources, were incubated for up to 48 h in a humidified atmosphere at 38°C. The embryos were staged by the number of somite pairs formed. Chick embryos at stages ranging from 15 to 20 somite pairs were used throughout this study. The somite staging system [16] has been used for numbering somites. In this system, the newly formed somite is referred to as somite S_I. We refer to somite S_0 as being the forming somite whose caudal clefts are not yet completely formed.

In vitro *culture and cycloheximide treatment of explants*

Microsurgical operations on the embryonic halves and *in vitro* culture were carried out as described [1]. The caudal portion of chick embryos was separated along the midline. The experimental half was incubated in the presence of 10 μM cycloheximide and the contralateral half was either cultured for the same period of time or fixed immediately. In all series, explants were processed for whole mount *in situ* hybridisation with *c-hairy1* or *lFng* probes. Efficiency of protein synthesis inhibition in these conditions has been previously established [1].

In situ *hybridisation*

The *c-hairy1* probe has been described [1]. The *lFng/c-Fringe1* probe was produced as described [15]. Whole-mount *in situ* hybridisation was performed as described [17]. Embryos were photographed as whole mounts using a Leica MZ10 stereomicroscope. *In situ* hybridisations on 20 μm cryosections were carried out as described [18].

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